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Purification and immunomodulating activity of C-phycocyanin from *Spirulina platensis* cultured using power plant flue gas

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ABSTRACT

In this study, flue gas from a power plant smokestack was applied to culture *Spirulina platensis* microalgae. Our results will not only achieve the fixation of carbon from the emissions, products can also be produced from the algal biomass that possess physiological activities which could be beneficial to human health. An improved one-step process of chromatography was used to produce high-purity C-phycocyanin with a PC ratios >3.5. Adding different concentrations of ammonium sulfate produced different amounts of C-phycocyanin, with 40% generating the highest yield, followed by 35% and 30% concentrations. Immunomodulating activities were evaluated in the murine macrophage cell line J774A.1. We found that C-phycocyanin had the capability to induce secretion of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and that these results were not due to contamination with LPS. Treatment with C-phycocyanin also increased proIL-1β and COX-2 protein expression dose-dependently. Furthermore, C-phycocyanin rapidly stimulated phosphorylation of inflammatory-related signaling molecules, including ERK, JNK, p38 and IκB. In addition, although C-phycocyanin decreased production of LPS-induced ROS, it did not inhibit LPS-induced inflammatory cytokines in J774A.1 cells. This is the first report to show that C-phycocyanin exhibited a detailed molecular mechanism of bioactivity by boosting immunomodulation performance.

1. Introduction

Because of the excessive discharge of global CO_2 emissions, we are facing an ever more serious greenhouse effect and other environmental problems. In addition to limiting CO_2 emissions within reasonable constraints, the conversion of carbon sources into usable chemicals or sustainable energy sources is becoming a focus of the utilization of global biomass resources. In our previous study which is using flue gas from a power plant smokestack to mass-culture *Spirulina platensis* microalgae in a photobioreactor [1]. This will not only achieve the fixation of carbon from the emissions products can also be produced from the algal biomass that possess physiological activities which could be beneficial to human health.

Since ancient times, *S. platensis* has been utilized as a food or food supplement for humans. In recent years, there has been a trend toward using the algal products in functional food items or as raw materials for cosmetic products. Phycocyanin, the active component of *S. platensis*, is a colorant present in cyanobacteria and red algae. Upon purification, phycocyanins show a brilliant blue color in solution. Phycocyanins are composed of two subunits α and β combined together [2–4]. In nature, it exists as monomers, trimers or hexamers; small quantities of oligomers have been found as well [5]. In general, phycocyanins include C-phycocyanin and allophycocyanin. They possess different maximum absorption peaks, 620 and 650 nm, respectively. Studies have suggested that the ratio of

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absorbance at 620 and 280 nm can be employed to indicate the purity of C-phycocyanin, while the ratio of absorbance at 650 and 280 nm can indicate the purity of allophycocyanin [6].

In addition, because of their special bioactivities they are increasingly recognized as potential raw materials for making food products that are beneficial to health [7]. Many studies have indicated that phycocyanins have bioactivities that include anti-tumor, antioxidant, and anti-inflammatory effects [8–13]. Several earlier studies indicated that C-phycocyanin has antioxidant and anti-inflammatory efficacies [12,14–16]. Other reports indicated that C-phycocyanin has anticancer bioactivity [17,18]. In 2003, a study by Reddy et al. demonstrated that C-phycocyanin could reduce the proliferation of macrophages (RAW 264.7) with increasing dosages [15]. In addition, C-phycocyanin was found to be able to selectively suppress COX-2 and PGE_2 expression when RAW 264.7 macrophages were stimulated by lipopolysaccharides (LPS), thus demonstrating the anti-inflammatory capability of C-phycocyanin.

Research on immunomodulation by natural products is a concern for anti-infective therapies and health care. It is well-known that macrophages play an important role in first-line immunological defense against host infections in mammals, and destroy tumor cells through the secretion of various cytokines [19]. In an immune response, the macrophages directly destroy foreign microorganisms and tumor cells by secreting a variety of cytokine mediators, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), etc. IL-1β can prompt the proliferation of T-cells, induce B-cells to produce antibodies, and boost the cytotoxic functions of CTL (cytolytic T lymphocytes) and NK (natural killer) cells. IL-6, on the other hand, stimulates the liver to produce acute-phase proteins, and stimulates B-cell proliferation; whereas TNF-α has direct cytotoxic and growth-suppression effects against tumor cells [20–22].

Several obstacles exist in the purification process of C-phycocyanin [23–25]. First of all, when algal yield is high, the C-phycocyanin purity tends to be low. However, if high purity C-phycocyanin is desired, the experimental procedure is rather complicated and entails a high production cost. Some studies have been carried out with phycocyanin, but there has often been a lack of elucidation of the cell molecular mechanism [26,27]. Thus, the main focus of the present study was on rapid purification of phycocyanin from *S. platensis* and analysis of its bioactivities.

2. Materials and methods

2.1. Chemicals and antibodies

LPS (from *E. coli* 0111:B4), polymyxin B (PMB), monoclonal anti-MAP kinase, activated (diphosphorylated ERK) antibody, monoclonal anti-JNK kinase, activated (diphosphorylated JNK) antibody, monoclonal anti-p38 MAP kinase, activated (diphosphorylated p38) antibody, monoclonal anti-actin antibody and commercial C-phycocyanin were purchased from Sigma–Aldrich (St. Louis, MO, USA). A mouse IL-1β, TNF-α and IL-6 ELISA (enzyme-linked immunosorbent assay) kit was purchased from R&D Systems (Minneapolis, MN, USA). Anti-IL-1β polyclonal antibody, anti-IκB-α antibody, anti-phospho-IκB-α antibody, anti-COX-2 antibody, anti-rabbit IgG-HRP, and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Extraction of crude phycocyanin from S. platensis

S. platensis microalgae were cultured in the three-dimensional photobioreactor at the Dalin power plant in southern Taiwan [1]. A simple repetitive freeze-and-thaw process was applied to degrade the cell walls of *S. platensis* microalgae.

2.3. Purification of pure C-phycocyanin from crude phycocyanin

Then, using ultrasonic vibratory treatment in PBS, crude C-phycocyanin was dissolved. In brief, 20 g (oven-dried) of *S. platensis* biomass was soaked at room temperature in 300 ml of PBS (1 L of PBS contained 137 mmol of NaCl, 2.7 mmol of KCl, 10 mmol of Na_2HPO_4, and 1.76 mmol of KH_2PO_4; pH = 7.4); then the solution was placed in an ultrasonic bath (Branson 5510), sonicated and extracted for 2 h. The solution was placed in a −80 °C freezer for 24 h, then deiced at room temperature and centrifuged using an ultracentrifuge at 4 °C (12,000 rpm, 30 min). To the supernatant, a series of $(NH_4)_2SO_4$ (30, 35 and 40%) were added separately to salt out the protein. After repeating 4 °C ultracentrifugation (12,000 rpm, 30 min), the precipitated protein fraction was re-dissolved with PBS and filtered through a 0.22 μm membrane. Dialysis was carried out using a Spectra/Por® membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA) with a molecular weight cutoff of 1 kDa. Water was replaced with fresh deionized water twice daily for 2 days. The resulting C-phycocyanin was then freeze-dried and quantified. Two hundred mg of crude C-phycocyanin (PC ratios ca. 0.8) was weighed and redissolved in PBS. Then a GPC 1122 liquid chromatography solvent delivery system (LC Tech, Dorfen, Germany) fitted with a HiPrep 26/60 Sephacryl™ S-300 high-resolution column (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used to purify C-phycocyanin. The conditions of isolation were: each injection amount was 5 ml, elution solution was 1 × PBS (containing 0.05% NaN_3), the flow rate of eluent was set at 3.0 ml/min, and 260 s per tube was collected. C-phycocyanin from the fifth tube on a UV-vis spectrophotometer (Agilent 8453; Agilent Technologies, Santa Clara, CA, USA) was used to determine the PC ratios (A_{620}/A_{280}) of the contents. For PC >2.0, the fraction was separately collected, then dialysis was repeated (Spectra/Por® membrane, molecular weight cutoff 1 kDa); every 12 h the water was replaced. After three exchanges, the post-dialysis phycocyanin was freeze-dried, collected separately and quantified. In order to increase the separation efficiency, the NaCl concentration gradient of the eluent added was from 0 mM to 30 mM.

2.4. Cell cultures

J774A.1 murine macrophages obtained from the Bioresource Collection and Research Center (Taiwan, ROC) were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA), and cultured at 37 °C in a 5% CO_2 incubator.

2.5. Cell toxicity of C-phycocyanin

Cell proliferation was determined using an MTT assay [28]. J774A.1 macrophages were seeded in 96-well plates at a density of 5×10^3 cells/well. Cells were incubated with C-phycocyanin (50–400 μg/ml) for 24 h. The value was determined by averaging triplicate sample measurements.

2.6. Western blotting analysis

Whole cell lysates or C-phycocyanin samples were separated by 12% SDS-PAGE and electrotransferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h, and then incubated at room temperature for 2 h with anti-proIL-1β antibody, anti-MAP kinase antibody, diphosphorylated ERK-1 and -2 antibody, anti-JNK kinase antibody, anti-p38 MAP kinase antibody, anti-COX-2 antibody, or anti-IκB antibody. After washing three times in PBS with 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody directed against the primary antibody. The membrane was developed by an enhanced chemiluminescence Western blotting detection system (DuPont NEN Research Products, Boston, MA, USA) according to the manufacturer's instructions [29].

2.7. Enzyme-linked immunosorbent assay (ELISA)

In the dose response study, J774A.1 cells (1×10^6/ml) were stimulated with C-phycocyanin only or with C-phycocyanin and LPS for determining TNF-α and IL-6 (after 6 h) and IL-1β (after 24 h). The concentration of cytokines in the conditioned medium was analyzed by ELISA according to the manufacturer's protocol, using Quantikine® mouse TNF-α, IL-6 and IL-1β immunoassay kits (R&D Systems) [30]. Biotinylated antibody reagent (50 μL) and 50 μL supernatant concentrate from samples tested for various times were added to anti-mouse TNF-α, IL-6 and IL-1β precoated stripwell plates, followed by incubation at room temperature for 2 h. After washing the plates three times with the washing buffer provided, 100 μL diluted streptavidin-HRP concentrate was added to each well and incubated at room temperature for 30 min. The washing process was repeated; then 100 μL premixed TMB substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition of 100 μL provided stop solution to each well to stop the reaction, the absorbance of the plates was measured by a Gemini EM microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) at 450–550 nm wavelengths.

2.8. Monitoring of LPS contamination of C-phycocyanin in experiments

Previous studies of C-phycocyanin-mediated reactions and signaling have encountered the problem of LPS contamination. Reagents and utensils for the preparation of C-phycocyanin were either LPS-free grade or were washed with PBS containing 10 μg/ml PMB, then rinsed with PBS. In order to rule out possible LPS contamination of C-phycocyanin samples, J774A.1 cells were pre-incubated with or without PMB (10 μg/ml) for 30 min, followed by treatment for 6 h with C-phycocyanin (400 μg/ml) or 24 h with LPS (0.03, 0.1, 0.3 or 1 μg/ml).

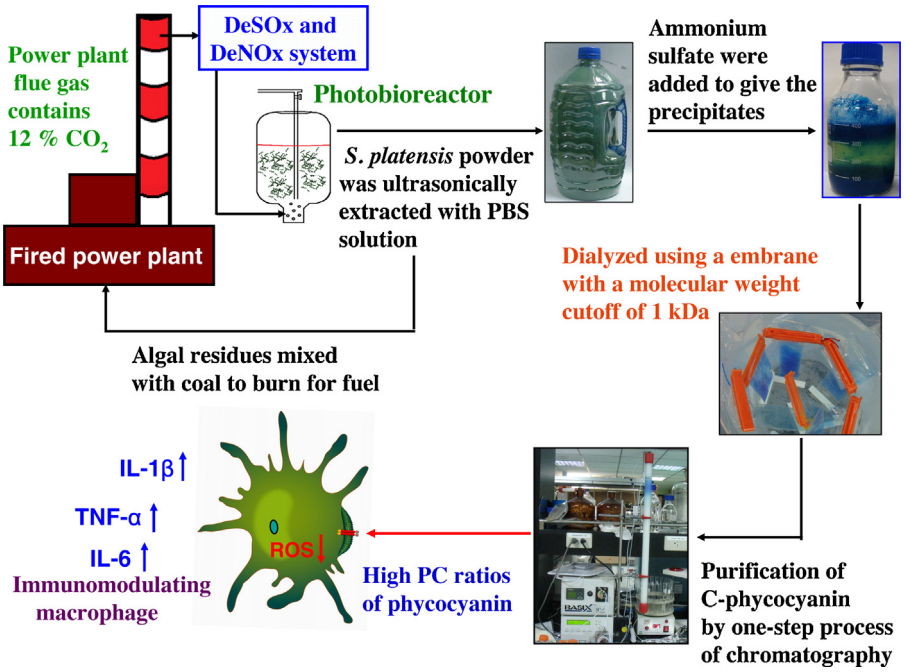


Fig. 1. The flow diagram of the procedure.

2.9. Antioxidant activity of C-phycocyanin

The antioxidant activity of C-phycocyanin was determined by intracellular H_2O_2 stimulated by C-phycocyanin, which was measured by detecting the fluorescence intensity of chloromethyl-2′,7′-dichlorofluorescin (CM-DCF), the oxidized product of chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCDA) (Molecular Probes, Eugene, OR, USA). Briefly, J774A.1 cells (1×10^6/ml) grown in serum- and phenol red-free RPMI medium for 24 h were then preincubated with CM-H_2DCDA (2 μM) and N-acetyl cysteine (NAC) (10 mM) at 37 °C for 30 min in the dark. This was followed by adding fresh starvation medium containing C-phycocyanin (50–400 μg/ml) or LPS (1 μg/ml) for an additional time, as indicated above. The relative fluorescence intensity of the fluorophore CM-DCF, which was formed by peroxide oxidation of the nonfluorescent precursor, was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm with a Gemini EM microplate spectrofluorometer (Molecular Devices).

2.10. Statistical analysis

All values are given as mean ±SD. Statistical analysis was performed by ANOVA for analysis and followed by suitable post hoc Duncan's multiple range test (DMRT) test was applied to calculate the statistical significance between various groups. A value of $P < 0.05$ was considered to be statistically significant.

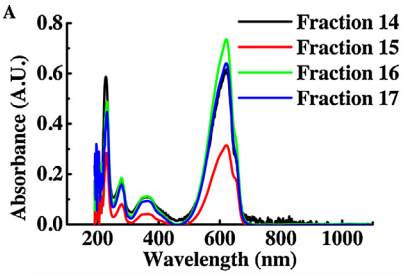
3. Results

3.1. Isolation and purification of C-phycocyanin

A notable feature of the present study was that the purification of C-phycocyanin was not as complicated as those described in earlier studies. The process merely involved dissolving C-phycocyanin and protein from *S. platensis* using phosphate buffered saline (PBS); then a 40% $(NH_4)_2SO_4$ solution was added to salt out crude C-phycocyanin. Upon filtration using a 0.22 μm membrane, the products were freeze-dried and quantified. A gel-filtration column was used to obtain pure C-phycocyanin with PC ratios >3.5. The flow diagram of the procedure is shown in Fig. 1.

Table 1 shows that by using different concentrations of ammonium sulfate, different yields of C-phycocyanin were obtained. The 40% group produced the highest yield. C-phycocyanin obtained with different ammonium sulfate concentrations were separately collected at PC fraction ratios of 2.5, 3.0, 3.5 and >3.5. The results indicated that the higher ammonium sulfate concentration, the greater C-phycocyanin yield. However, regardless of ammonium sulfate concentrations, the higher PC ratios of the fraction, the lower yield became. In addition, Table 1 also shows the average precipitation yields of C-phycocyanin respectively with 30%, 35%, and 40% ammonium sulfate solution, which were respectively 6.54%, 8.37%, and 15%. The average fractional yields (in mg) of C-phycocyanin with different PC ratios are shown at the lower right corner of Table 1.

The spectra and absorbance values of different purified C-phycocyanin fractions collected are showed in Fig. 2A. After fraction 17, the PC ratios decreased rapidly to less than 2.5. Test specimens collected after fraction 14 were subjected to SDS electrophoresis (Fig. 2B). In the figure, line 1 was from the standard purchased from Sigma–Aldrich, which had a PC ratios >3.5. Line 2 was our purified C-phycocyanin, having a PC ratio of 3.51. Fig. 2A and B was to prove that the C-phycocyanin obtained in the study was not merely proven using the PC ratios, but the SDS-PAGE was engaged to prove that the purity of C-phycocyanin was similar to the purchased high-purity product from Sigma Chemicals; that the α and β subunits of



	280 nm	620 nm	620/280 ratio
Fraction 14	0.174	0.612	3.513
Fraction 15	0.080	0.314	3.907
Fraction 16	0.183	0.735	4.009
Fraction 17	0.157	0.640	4.070

Fig. 2. Purity of C-phycocyanin from *Spirulina platensis*. (A) UV–vis spectra and PC ratios of different fractions of crude C-phycocyanin after liquid chromatographic purification using a Sephacryl™ S-300 high-resolution column. (B) SDS-PAGE of purified C-phycocyanin (fraction 14) vs. standard (Sigma–Aldrich), both with PC ratios >3.5.

Table 1
C-phycocyanin (C-PC) yields (%) and the weights of collected from different PC ratios by using different ammonium sulfate concentrations.

	PC ratios (A_{620}/A_{280})			
	2.5	3.0	3.5	>3.5
30% $(NH_4)_2SO_4$, C-PC avg. yields (6.54%)	9.0 mg	7.25 mg	1.87 mg	0.5 mg
35% $(NH_4)_2SO_4$, C-PC avg. yields (8.37%)	12.5 mg	4.75 mg	3.25 mg	0.75 mg
40% $(NH_4)_2SO_4$, C-PC avg. yields (11.35%)	15.0 mg	5.75 mg	5.0 mg	1.75 mg

Note: Each injection was 5 ml; the crude C-phycocyanin dissolved was ca. 200 mg. The yield must be multiplied by the original ammonium sulfate-precipitated *S. platensis* C-phycocyanin average yield (in parentheses), which provides the actual C-phycocyanin yield. Average yields of *S. platensis* C-phycocyanin from ammonium sulfate precipitation were based on at least five replications. The average yields for different PC values were based on 10 replications after LC purification.

C-phycocyanin had identical positions and we have remarked the α and β subunits of C-phycocyanin in Fig. 2B. These experimental results indicated that using the experimental procedure produced reliable high-purity C-phycocyanin.

3.2. Cytotoxicity and immunomodulation activity of C-phycocyanin

Prior to investigating the bioactivity of C-phycocyanin, an MTT assay was used to test for cytotoxicity. The results revealed that when C-phycocyanin (400 μg/ml) or LPS (1 μg/ml) were added to cells, followed by incubation for 24 h, no cell death occurred. The particular dosage of both C-phycocyanin (25–400 μg/ml) and LPS had no bearing on cytotoxicity (Fig. 3A).

Infection by pathogenic bacteria will cause inflammatory cytokines such as TNF-α, IL-1β and IL-6 or their modulating factors to increase, which in severe cases may lead to the death of patients. Previous studies have shown that C-phycocyanin contains anti-inflammatory capability in LPS-stimulated RAW 264.7 macrophages [15,31]. In order to examine whether C-phycocyanin purified from *S. platensis* has anti-inflammatory activity in murine J774A.1 macrophages during an infection process, we first added different doses of C-phycocyanin to macrophages, incubated them for 30 min, and then added LPS (1 μg/ml) followed by incubation for 6–24 h. Fig. 3B–D shows that in murine macrophages pretreated

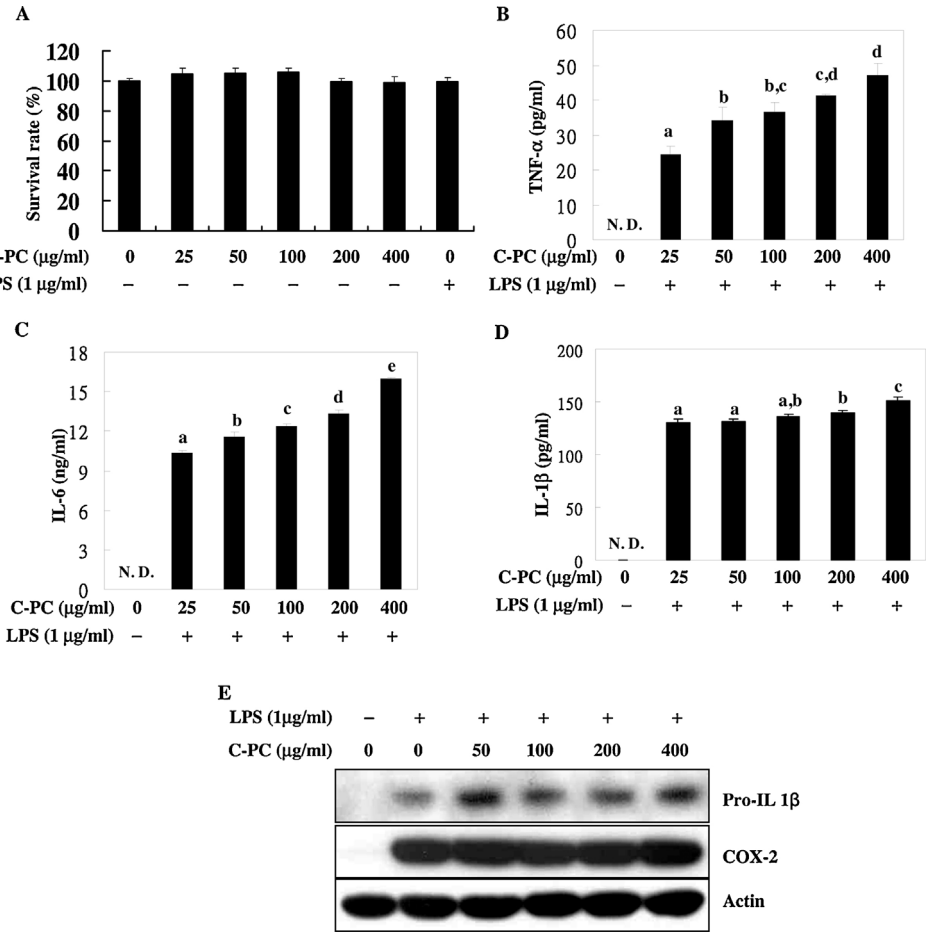


Fig. 3. Cytotoxicity and effects of C-phycocyanin on LPS-stimulated inflammatory mediators. (A) Cell toxicity of f C-phycocyanin (C-PC) was assayed by Alarmar Blue method. (B) and (C) J774A.1 macrophages subjected to treatment with C-phycocyanin (C-PC, 0–400 μg/ml) for 30 min, followed by stimulating with 1 μg/ml of LPS for 6 h. Secreted amount of TNF-α (B), IL-6 (C) in culture medium was assayed by ELISA. (D) Secreted amount of IL-1β from J774A.1 macrophages subjected to treatment with C-phycocyanin (C-PC, 0–400 μg/ml) for 30 min, then with 1 μg/ml of LPS followed by incubating for 24 h. The cytokine concentration in culture medium was assayed by ELISA method. Data are expressed as mean ± SE from three separate experiments. (E) J774A.1 macrophages (1×10^6/ml) were pretreated with 0–400 μg/ml of C-phycocyanin (C-PC) for 30 min, followed by stimulating with 1 μg/ml of LPS for 6 h. The expression of pro-IL1β and COX-2 was assayed by Western blot. The result of one of three separate experiments is shown.

with C-phycocyanin, there was no significant suppression of the expression of TNF-α, IL-1β and IL-6. In LPS-stimulated cells, the secretion amount of TNF-α was 24 ng/ml. Under pretreatment conditions using 50, 100, 200 and 400 μg/ml of C-phycocyanin, however, the ensuing TNF-α was 34, 36, 41 and 47 ng/ml, respectively (Fig. 3B). LPS stimulation alone caused IL-6 secretion of 10 ng/ml, whereas with pretreatments of 50, 100, 200 and 400 μg/ml of C-phycocyanin, the secretion of IL-6 rose to 11, 12, 13 and 16 ng/ml (Fig. 3C). From stimulation by LPS alone, IL-1β expression was 130 pg/ml; whereas after pretreatment with the aforementioned C-phycocyanin doses, the expression levels increased to 131, 135, 139 and 151 pg/ml, respectively (Fig. 3D). We also used western blotting to test protein expression of inflammatory-related proteins, include proIL-1β and COX-2. As shown in Fig. 3E, J774A.1 macrophages were pretreated with 0–400 μg/ml of C-phycocyanin for 30 min; then cells were stimulated with 1 μg/ml of LPS, followed by incubation for 6 h. The results indicated that there was no discernible suppression of the expression of proIL-1β and COX-2 as stimulated by LPS.

These results differed from those of Cherng et al. [31], which showed that in a RAW 264.7 (murine microphage) cellular model, the presence of C-phycocyanin could significantly suppress the expressed amount of iNOS and NF-κB activation stimulated by LPS (120 μg/ml). Their results indicated that C-phycocyanin has the capacity to suppress production of cytokines when murine RAW 264.7 macrophages were stimulated by LPS. Because our data differed from a previous study [31], we next investigated whether C-phycocyanin contains other bioactivity, such as immunomodulation. To examine the immunomodulation activities of C-phycocyanin, J774A.1 macrophages were treated with 0–400 μg/ml of C-phycocyanin for 6 h; the secretion of cytokines in the medium was tested using an ELISA method. To exclude the possibility of LPS contamination, cells were co-treated with PMB, a cyclic amphipathic peptide antibiotic that binds to endotoxins. As shown in Fig. 4A, the secreted amount of TNF-α increased with increasing amounts of C-phycocyanin. However, even after adding 10 μg/ml of PMB, the expressed amount of TNF-α boosted by C-phycocyanin was not totally suppressed. Fig. 4B shows the effects of adding 50–400 μg/ml C-phycocyanin on the production of IL-6 by the J774.1 cells. The trend was similar to that in Fig. 4A. The effect of C-phycocyanin stimulation of J774.1 macrophages on the expression of IL-1β is shown in Fig. 4C. Notably, at a 50 μg/ml dose, there was no IL-1β production; but at 100–400 μg/ml C-phycocyanin doses there was significant activation of IL-1β secretion. Additionally, when only C-phycocyanin (400 μg/ml) without LPS was added to J774A.1 macrophage culture, the expression of COX-2 was increased dose-dependently; while proIL-1β expression was only detected after adding a 400 μg/ml dose of C-phycocyanin (Fig. 4D).

When added alone, a 400 μg/ml dose of C-phycocyanin caused only a mild expression of TNF-α (Fig. 4A), unlike that induced by a general pathogen (antigen) that releases large amounts of inflammatory cytokines. In other words, the expressed amount was not very high: less than half of what 0.03 μg/ml of LPS could induce (Fig. 4E). Therefore, C-phycocyanin evidently possesses bioactivity, i.e. the ability to modulate immune cells in mammals. Cytokines such as IL-1ß could prompt the proliferation of T-cells, induce B-cells to produce antibodies, and boost the cytotoxic functions of CTL (cytolytic T lymphocytes) and NK (natural killer) cells. IL-6 stimulates the liver to produce acute-phase proteins and B-cell proliferation; whereas TNF-α has direct cytotoxic and growth-suppression effects against tumor cells.

These results, however, differed somewhat from the findings of previous reports [15,31]. In their study, the C-phycocyanin was from Sigma–Aldrich and the PC was >3.5. Therefore, in this study a comparison was made of the differences in bioactivities between commercial C-phycocyanin (purchased from Sigma–Aldrich) and C-phycocyanin purified from *Spirulina* grown in a photobioreactor. First of all, the commercial C-phycocyanin was a liquid with fair amount of salts, including EDTA and PBS (155 mM). In addition, after adding commercial C-phycocyanin alone (50 μg/ml) the J774A.1 murine macrophages had only a 75% survival rate (data not shown). If the C-phycocyanin (50 μg/ml) was added 30 min before LPS stimulation, the cellular survival rate increased to 89%. Conversely, when C-phycocyanin purified from *Spirulina* was added at 400 μg/ml dose, there was no cell death. Apparently, without dialysis, the commercial C-phycocyanin preparation might be slightly cytotoxic. At the same dose (50 μg/ml), however, we found no apparent suppression effect on the production of TNF-α by J774A.1 murine macrophages when commercial C-phycocyanin was used (data not shown).

3.3. Monitoring of LPS contamination of C-phycocyanin in experiments

In order to confirm the immunomodulating effects of C-phycocyanin and ascertain that the purified C-phycocyanin from *S. platensis* was not contaminated with LPS, a set of experiments was designed to prove the authenticity of the experimental results. As shown in Fig. 4E, LPS was diluted separately to 0.03, 0.1, 0.3 and 1 μg/ml to stimulate the J774A.1 macrophages; then 10 μg/ml of PMB (a polypeptide antibiotic with a positive charge, which is often used to suppress the cytokines stimulated by LPS in in vitro studies) was added separately to suppress the expression of the inflammatory cytokine TNF-α. The results indicated that 10 μg/ml of PMB could totally suppress the production of TNF-α at LPS doses of 0.03, 0.1 and 0.3 μg/ml; however, it failed to totally suppress the expression of TNF-α after a 1 μg/ml dose of LPS (the expressed amount was 200 pg/ml). When cells were stimulated by C-phycocyanin (400 μg/ml), the expression of TNF-α increased to 300 pg/ml; and after the further addition of 10 μg of PMB, the TNF-α decreased only slightly, to 250 pg/ml. These results clearly show that the TNF-α produced by C-phycocyanin was affected only minimally by PMB. This may primarily be due to the charged nature of the protein. If the C-phycocyanin contained an extremely low concentration of LPS, then its existence in the C-phycocyanin must be >0.075%. Even if this were true, then 10 μg/ml of PMB can totally suppress the expression of TNF-α. Therefore, the experimental results should be construed as proof that our C-phycocyanin was totally free from contamination by LPS, and that the results were a manifestation purely of C-phycocyanin induction on the J774A.1 macrophages.

3.4. Activation of MAPK and NF-κB signaling pathways by C-phycocyanin

MAPKs and NF-κB signaling are involved in cytokine secretion and proinflammatory molecule expression in macrophages. A dosage of 400 μg/ml of C-phycocyanin applied to J774A.1 cells appeared to activate the mitogen-activated protein kinases (MAPKs) ERK, JNK and p38 by phosphorylation. After 10 and 60 min of treatment, phosphorylation of IκB was detected. As is well known, after phosphorylation, IκB will be degraded by proteasomes; this allows NF-κB to enter the nucleus. The results of this experiment suggested that C-phycocyanin indeed possesses bioactivity, and is able to modulate murine macrophages (Fig. 5).

3.5. Antioxidant activity of C-phycocyanin

A previous study has demonstrated that C-phycocyanin contains antioxidant properties [10]. Fig. 6 shows the antioxidant effects of C-phycocyanin-pretreated J774A.1 cells. LPS stimulation of cells rapidly induces ROS production when compared with that of control cells. In contrast, pretreatment with N-acetylcysteine (NAc), a

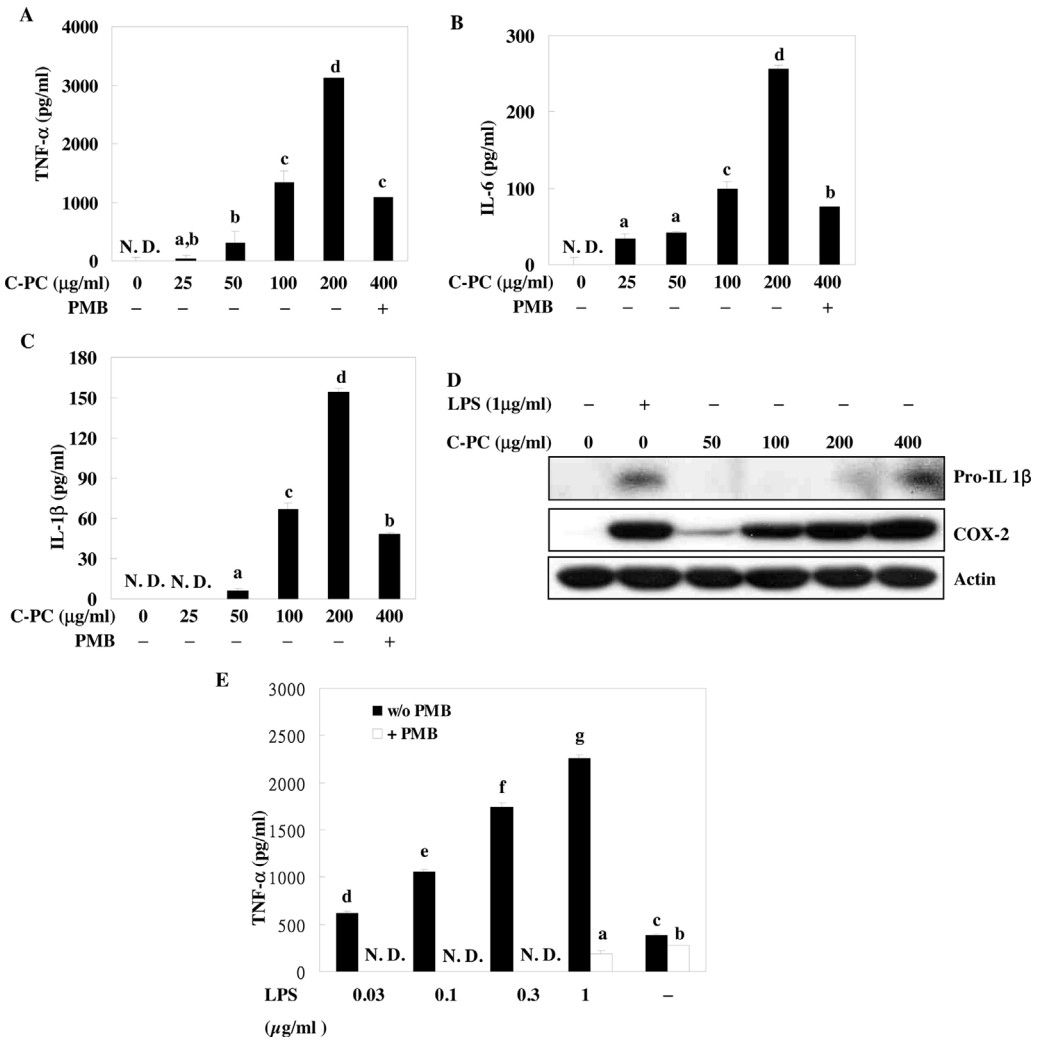


Fig. 4. Effect of C-phycocyanin (C-PC) on the expression of inflammatory mediators. (A)–(C) J774A.1 macrophages were treated with C-phycocyanin (C-PC, 0–400 μg/ml) for 6 h or 24 h in the absence or presence of 10 μg/ml PMB. TNF-α (A), IL-6 (B), IL-1β (C) concentration in culture medium was assayed by ELISA. Data are expressed as mean ± SE from three separate experiments. (D) J774A.1 cells were treated with 0–400 μg/ml of C-phycocyanin (C-PC) for 6 h. Cells lysates were separated by 10% SDS–PAGE and immunoblotted with anti-pro-IL1β and anti-COX-2. Shown are representative blots of at least three repeats at all data points. (E) J774A.1 cells were pretreated with LPS (0.03–1 μg/ml) or C-phycocyanin (C-PC, 400 μg/ml) for 6 h in the absence or presence of 10 μg/ml PMB. The TNF-α concentration in culture medium was measured by ELISA.

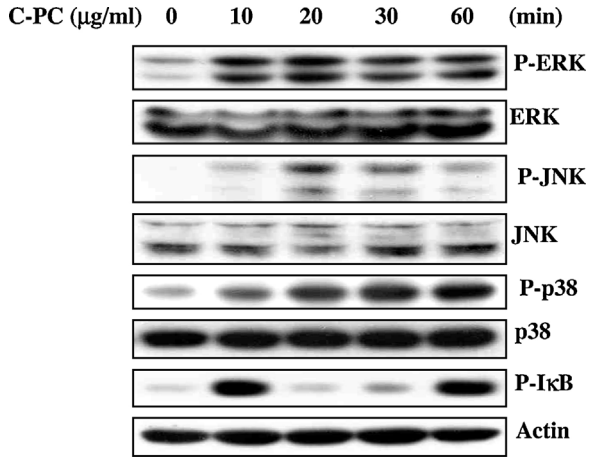


Fig. 5. Effect of C-phycocyanin on MAPKs phosphorylation and NF-κB activation. J774A.1 cells were treated with C-phycocyanin (C-PC, 400 μg/ml) for 0–60 min. The phosphorylation levels of ERK1/2, JNK1/3 and p38 were analyzed by Western blot. Data are from one of the representative experiments ($n = 3$).

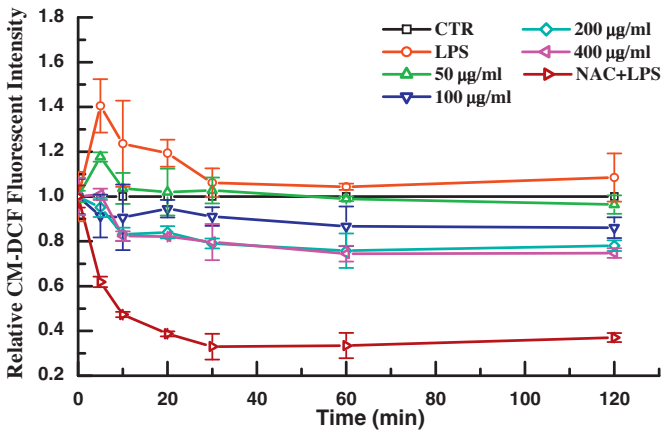


Fig. 6. C-phycocyanin inhibited ROS release in J774A.1 macrophages stimulated with LPS (1 μg/ml). Cells were pretreated with H_2DCFDA (2 μM) for 30 min, followed by substitution with fresh medium. Cells were treated with C-phycocyanin (C-PC, 0–400 μg/ml) or with N-acetyl cysteine (NAC) (10 mM) for 30 min, prior to incubation with LPS (1 μg/ml) for 0–60 min. The relative fluorescence intensity of fluorophore CM-DCF was detected as described in Section 2. Data are expressed as mean ± SEM of three independent experiments.

potent antioxidant, quickly reduces the production of LPS-induced ROS. Along with increasing dosages of C-phycocyanin, the ROS (H_2O_2) content in cells was decreased within 2 h.

4. Discussion

In Table 1, our results demonstrated that higher concentration of ammonium sulfate resulted in higher yields of C-phycocyanin of higher PC ratios. Comparing the 40% and 30% ammonium sulfate, the yield gain was less than 2-fold (11.35% vs. 6.54%), however, the fraction of C-phycocyanin with PC ratios >3.5 were 1.75 mg vs. 0.5 mg; for product of PC ratio = 3.5, the yields were 5.0 mg vs. 1.87 mg. Apparently, the high concentration of ammonium sulfate was an important factor in favoring products of high PC ratios (i.e., produced higher yield and greater purities). This experimental study showed that C-phycocyanin isolated from *S. platensis* microalgae possesses immunomodulatory activity. In this study the experimental results proved that purified C-phycocyanin induced secretion of immune cytokines such as TNF-α, IL-1β and IL-6, and regulated intracellular proteins such as proIL-1β, COX-2, phosphorylated Iκβ, and MAPK in J774A.1 murine macrophages. In addition, C-phycocyanin contained antioxidant activity. However, C-phycocyanin produced no apparent anti-inflammatory bioactivity in a J774A.1 murine macrophage model.

In our previous study, a novel photobioreactor was developed which is utilizing CO_2 in the flue gas of a power plant as the carbon source for the growth of a seawater alga, *S. platensis*. This will not only accomplish the fixation of carbon from the emissions; products can also be produced from the algal biomass that possesses physiological activities. Otherwise, microbial decomposition will quickly return the fixed carbon back into the atmosphere. Additionally, in the process of treating biomass for reutilization, the necessary energy requirements and ensuing CO_2 emissions must be taken into account in order to avoid overloading the environment. Nevertheless, the culturing of algal biomass is likely to have a positive overall significance in carbon sequestration. Regardless of whether the biomass is used as a health food supplement or as animal feed, carbon will be fixed in biological entities as a consequence. Therefore, further efforts toward understanding the bioactivities of such biomass are warranted.

Many studies have traditionally used the ratio of absorbance at 620 and 280 nm to express the purity of C-phycocyanin. In a paper by Piñero Estrada et al., a hydroxyapatite column was first used to purify phycocyanin, and then a DEAE Sephadex A-50 (Sigma–Aldrich) gel-filtration column to further purify the product [32]. They obtained a product with an A_{620}/A_{280} nm ratio of approximately 3.9. Other studies have used an absorbance ratio of A_{615}/A_{280} nm as an indication of C-phycocyanin purity [7]. However, their reported C-phycocyanin purities were all quite low (>0.5).

Chaiklahan et al. successfully utilized a membrane process to extract phycocyanin en masse from a *Spirulina* sp. using ultrafiltration. Although the procedure produced larger quantities of phycocyanin, the PC purity value was only ca. 1.07, and thus not very high [4,33]. Unlike some prior studies, which generally were run at a 50% concentration of ammonium sulfate, relatively lower concentrations were used. In the present study it was found that with increasing ammonium sulfate concentration, the yield of crude phycocyanin tended to increase; however, non-target motley proteins of other origin also increased in the solution. Thus, although the total protein extraction increased, the purification process became more complicated as well. As a consequence, a 40% concentration of ammonium sulfate was used to precipitate crude C-phycocyanin. Ammonium sulfate solutions of 30 and 35% tended to produce too low a yield, however.

In this study a murine macrophage cell line, J774A.1, was employed as an experimental model. The results showed that the C-phycocyanin purified from *S. platensis*, when applied to a murine J774A.1 macrophage model, exhibited bioactivity by boosting immunomodulation performance, and had the capability of inducing the expression of TNF-α, IL-1β, IL-6, proIL-1β and COX-2 in macrophage cells, as well as the phosphorylation of ERK, JNK, p38 and IκB. However, the results appeared to deviate from those of Cherng et al. [31]. They showed that in a RAW264.7 (murine microphage) cellular model, the presence of C-phycocyanin could significantly suppress the expressed amount of iNOS stimulated by LPS (120 μg/ml). In their study, the C-phycocyanin was from Sigma–Aldrich and the PC was >3.5. They also noted that 1 h after C-phycocyanin treatment at doses of 120–250 μg/ml, the expressed amount stimulated by LPS also showed significant suppression; however, C-phycocyanin treatment had no suppressive effect on IL-1β. When LPS was added alone, IκB-α was degraded significantly; however, when C-phycocyanin was also added, the degradation could be prevented. Their results indicated that C-phycocyanin has the capacity to suppress production of cytokines when murine macrophage cells were stimulated by LPS. One possible cause of differences in the experimental results might originate from the source of C-phycocyanin proteins. The C-phycocyanin used in their study was from Sigma–Aldrich, while in the present study C-phycocyanin was purified from *Spirulina* grown in a photobioreactor. Commercial C-phycocyanin shows slightly cytotoxicity and has no suppression effect on the production of TNF-α by J774A.1 murine macrophages (data not shown). In addition, there may be several probable causes for the discrepancy, including different cellular strains. Cherng et al. [31] and Reddy et al. [15] employed RAW 264.7 murine macrophages and different LPS. (They applied an *E. coli* 026:B6 strain, while in the present study an *E. coli* 0111:B4 strain was used.)

Because macrophage cells exist widely in various organs and tissues of the human body, they play a vital role in immune defense, including participating in immune response and inflammatory response, and maintaining the stability of the internal cellular environment. However, not all these functions are executed or expressed by macrophage cells simultaneously. Simply put, there exists among macrophage cells certain heterogeneity. The probable cause of this phenomenon is that macrophages are derived from different precursor cells which originally are situated in different growth environments [34]. In addition, different strains of macrophage cells might differentiate because of their varying genetic features [35]. Certain macrophage cells have an expressive nature disassociated from environmental factors. Conversely, they tend to react differently to certain specific stimulus cells. This is because different stimulating factors will cause activation of different intracellular signal transduction, resulting in macrophage cell heterogeneity.

Several signal transduction cascades are involved in the regulation of inflammatory mediator expression, such as MAPKs and NF-κB. Few studies have been conducted on the effects of phycocyanins on MAPKs, especially with respect to immunological cells. The present results indicated that such cells treated with C-phycocyanin will show activation of ERK, JNK and p38 protein expressions. In a study in a melanoma B16F10 cellular model, adding C-phycocyanin alone could increase the expression of ERK, but at the expense of suppressed p38 expression [36]. Nishanth et al. [37] found that C-phycocyanin added to hepatoma cells could suppress the content of reactive oxygen species (ROS) and COX-2, but did not affect the expression of ERK, JNK and p38 proteins.

ROS play important roles in LPS-mediated cytokine expression. In this study, we found that C-phycocyanin contains anti-oxidant activity. Our results are consistent with a previous study [9,10], although different cell strains and sources of C-phycocyanin were

used. It is generally accepted that ROS has an inflammatory effect, and its overproduction can cause functional chaos in cells and organs. A study by Zmijewski et al. [38], however, indicated that peroxides somehow have an anti-inflammatory effect. Through observation of the suppression of peroxidase or reduction in the induced inflammatory response by lipopolysaccharides, they explained the mechanism whereby increased peroxides in cells cause acute inflammation. The results of these studies suggest that when the immune system is boosted, it is necessary for ROS in cells to increase as well.

In conclusion, a moderate stimulation of macrophages by C-phycocyanin can increase the immunological activity of cells and allow the biological entity to obtain the benefits. As far as we know, no other paper has studied the intracellular molecular mechanisms of C-phycocyanin activities in such detail.

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